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Stanford MFEL and Near Infrared Science Center

Abstract

The Stanford MFEL and Near Infrared Science Center, through its support from the Air Force Office of Scientific Research (AFOSR), developed an interdisciplinary and collaborative program to advance military medicine in several areas of critical importance. The program has developed advanced surgical tools that will enable more precise procedures with less collateral damage such that wounds heal faster with less scarring. This advance is complemented by sophisticated analyses of laser and thermal damage to tissues that identified key regulators of the healing process, now being developed as therapies in skin and muscle as well an in the eye. The ophthalmology aspects of this program have developed technologies for restoration of sight and prevention of tissue damage. Similarly the projects on hearing have led to new tools for assessing hearing loss and guiding procedures for restoration of hearing—cochlear implants. Multifaceted approaches have been taken to understand the molecular and cellular basis of tissue damage and develop sophisticated means of tissue repair and restoration of function, including recruitment of resident stem cells in regenerative medicine. The teams consisted of biologists, engineers, physicists, and chemists such that many areas of investigation with a diversity of disciplines could be addressed.

1. Vibrational Microspectroscopy

a) Significant Accomplishments:

This project was centered on imaging of living cells and tissues with intrinsic chemical contrast. This approach allows avoiding exogenous staining often affecting cellular metabolism. We started development of this technology with near-field infrared imaging, using scanning probes and solid immersion microlenses. Later we developed a novel approach to fast near-field IR imaging using transient optical elements based on photo-induced reflectivity in semiconductors. To allow vibrational spectroscopy and imaging in liquid medium we also developed a coherent anti-Stokes Raman scattering (CARS) microscopy system. The coherent nature of this process can greatly enhance CARS signal compared to that of spontaneous Raman scattering. We have developed and tested a unique approach to CARS imaging that does not require scanning, and therefore is compatible with any standard microscope. It is called wide-field CARS microscopy, which provides the simultaneous imaging of an extended illuminated area without scanning. This method is based on the non-phase-matching illumination of a sample and imaging of a CARS signal with a CCD camera using conventional microscope optics. Imaging of neural tissues and water-immersed samples proved high (diffraction limited) spatial resolution and chemical selectivity of this technique.

b) Peer-Reviewed publications (in reversed chronological order):

On Illumination Schemes for Wide-Field CARS Microscopy. I. Toytman, D. Simanovskii, and D. Palanker, *Optics Express*, **17(9)**: 7339-7347 (2009).

Wide-field coherent anti-Stokes Raman scattering microscopy with non-phase-matching illumination. I. Toytman, K. Cohn, T. Smith, D. Simanovskii, and D. Palanker, *Optics Letters*, **32 (13)**: 1941-1943 (2007).

Transient Optical Elements: Application to Near-Field Imaging. D. Simanovskii, D. Palanker, K. Cohn, T. Smith, *J. Microscopy* **210(3)**: 307-310 (2003).

Transient photoinduced diffractive solid immersion lens for infrared microscopy. K. Cohn, D. Simanovskii, T. Smith, and D. Palanker, *Applied Physics Letters*, **81(19)**: 3678-3680, 2002.

Near-Field Infrared Microscopy With A Transient Photo-Induced Aperture. D. Simanovski, D. Palanker, K. Cohn and T. Smith, *Applied Physics Letters,* **79(8)**: 1214-1216, 2001.

Refraction Contrast Imaging With A Scanning Microlens, D.A. Fletcher, K.B. Crozier, C.F. Quate, G.S. Kino, and K.E. Goodson, D. Simanovskii, D.V. Palanker, *Applied Physics Letters,* **78(#23)**: 3589-3591, 2001.

On Contrast Parameters and Topographic Artifacts in Near-Field Infrared Microscopy, D.V. Palanker, D.M. Simanovskii, P. Huie, T.I. Smith, H.A. Schwettman, *Journal of Applied Physics,* **88(11)**: 6808-6814 2000.

Near-field infrared imaging with a microfabricated solid immersion lens, D.A. Fletcher, K.B. Crozier, C.F. Quate, G.S. Kino, and K.E. Goodson, D. Simanovskii, D.V. Palanker, *Applied Physics Letters,* **77(14)**: 2109-2111, 2000.

Etched Chalcogenide Fibers for Near-Field IR Scanning Microscopy, M.A. Unger, D.A. Kossakovski, R. Kongovi, J.L. Beauchamp, D.V. Palanker, *Review of Scientific Instruments,* **69(8**): 2988-93, (1998).

Fast IR Imaging with Sub-Wavelength Resolution using a Transient Near-field Probe, D. V. Palanker, G.M.H. Knippels, T.I. Smith, H.A. Schwettman, *Nuclear Instruments and Methods in Physics, Section B: Beam Interactions with Materials and Atoms* **144**: 240-245 (1998).

IR Microscopy with a Transient Photo-induced Near-field Probe (Tipless Near-field Microscopy), D. V. Palanker, G.M.H. Knippels, T.I. Smith, H.A. Schwettman, *Optics Communications,* **148/4-6** : 215 - 220 (1998).

c) Conference Proceedings Publications:

1. Solid state lasers for wide-field CARS microscopy, D. Simanovskii, I. Toytman, D. Palanker, *Solid State Lasers XVIII: Technology and Devices*, *SPIE,* vol. 7193 (2009).
2. Non-Scanning CARS Microscopy Using Wide-Field Geometry, I.Toytman, K.Cohn, T. Smith, D. Simanovskii, D. Palanker, *Multiphoton Microscopy in the Biomedical Sciences VII, SPIE,* vol. **6442** (2007).
3. On Image formation in Near-field Infrared Microscopy, D. M. Simanovskii, D. V. Palanker, P. Huie, T.I. Smith, *Scanning and Force Microscopies for Biomedical Applications II*, SPIE, vol. **3922**, (2000).
4. Fast IR Imaging with Sub-Wavelength Resolution Using a Transient Near-Field Probe (Tipless Near-Field Microscopy), D.V. Palanker, T.I. Smith, H.A. Schwettman, *Three Dimentional and Multidimensional Microscopy, SPIE, vol. **3605**, (1999).*

d) Agency or corporate funding spawned by project accomplishments:
None

e) Patents:
None

2. Beth Israel Deaconess Medical Center (Harvard) Subcontract: Optical Detection of Physiological Stress using Light Scattering Spectroscopy

a) Significant Accomplishments:
Fast and noninvasive detection of cellular stress is extremely useful for fundamental research and practical applications in medicine and biology. We discovered that light scattering spectroscopy enables monitoring the transformations in cellular organelles under thermal stress. At the temperatures triggering expression of heat shock proteins, the refractive index of mitochondria

increases within 1 min after the onset of heating, indicating enhanced metabolic activity. At higher temperatures and longer exposures, the organelles increase in size. This technique provides an insight into metabolic processes within organelles larger than 50 nm without exogenous staining and opens doors for noninvasive real-time assessment of cellular stress.

b) Publications (in reversed chronological order):

Optical Spectroscopy Non-Invasively Monitors Response of Organelles to Cellular Stress. G. Schuele, E. Vitkin, P. Huie, C. O'Conell-Rodwell, D. Palanker, L.T. Perelman. *J. Biomedical Optics*, **10(5)**: 051404-1 - 051404-8 (2005).

c) Conference Proceedings Publications:

Noninvasive Dosimetry and Monitoring of TTT using Spectral Imaging. G. Schuele, F.E. Molnar, D. Yellachich, E. Vitkin, L.T. Perelman, D. Palanker. *Ophthalmic Technologies XVI,* SPIE vol. **6138** (2006).

Optical monitoring of thermal effects in RPE during heating. G. Schuele, P. Huie, D. Yellachich, F. Molnar, C. O'Conell-Rodwell, E. Vitkin, L. T. Perelman, D. Palanker. *Ophthalmic Technologies XV,* SPIE vol.**5688A** (2005).

Non-invasive Monitoring of the Thermal Stress in RPE Using Light Scattering Spectroscopy. G. Schuele, P. Huie, A. Vankov, E. Vitkin, H. Fang, E.B. Hanlon, L.T. Perelman, D. Palanker, *Ophthalmic Technologies,* vol.**5314,** SPIE (2004).

d) Agency or corporate funding spawned by project accomplishments:

None

e) Patents:

None

3. **Laser-Tissue Interactions**

a) Significant Accomplishments:

We studied a wide range of laser-tissue interactions including photo-thermal, photo-mechanical, optical breakdown effects, and associated accompanying phenomena of cavitation, liquid flow and heat transfer in various biological tissues. In the field of laser surgery with ultrashort pulses, we developed two novel approaches to enhancement of the cutting rate of transparent biological tissues: the first approach is based on interactions of cavitation bubbles produced simultaneously in several focal points of a multifocal system. Second approach uses axially extended focusing system based on axicon, which allows producing a zone of dielectric breakdown with aspect ratio exceeding 500:1. We have demonstrated precise cutting of the bone with ultrashort pulse laser (800 nm), and accelerated healing of such cuts, compared to conventional mechanical devices. We developed laser control over release of proteins encapsulated into liposomes for controlled drug delivery. We established parameters for Arrhenius model of cellular thermal damage, which allowed predicting the size of the damage zone in tissue. In addition, we developed micofluidic devices driven by pulsed energy deposition for fluid injection.

b) Peer-Reviewed publications (in reversed chronological order):

Optical breakdown in transparent media with adjustable axial length and location. I. Toytman; D. Simanovski; D. Palanker. *Optics Express*. **18(24)**: 24688-24698 (2010).

Multi-Focal Laser Surgery: Cutting Enhancement by Hydrodynamic Interactions Between Cavitation Bubbles. I. Toytman, A. Silbergleit, D. Simanovski, D. Palanker. *Physical Review E* (2010)

Laser-induced disruption of systemically administered liposomes for targeted drug delivery; Mark A. Mackanos, Malika Larabi, Rajesh Shinde, Dmitrii M. Simanovskii, Samira Guccione, Christopher H. Contag; J. Biomed. Opt., Vol. 14, 044009 (2009);

Role of HSP70 in cellular thermotolerance. Beckham JT, Wilmink GJ, Mackanos MA, Takahashi K, Contag CH, Takahashi T, Jansen ED (2008) **Lasers Surg Med**, 40(10):704-715.

In-vivo optical imaging of hsp70 expression to assess collateral tissue damage associated with infrared laser ablation of skin. Wilmink, GJ, Opalenik, SR, Beckham, JT, Mackanos, MA, Nanney, LB, Contag, CH, Davidson, JM, Jansen, ED (2008) **J Biomed Optics** . 13(5): 054066.

In vivo analysis of heat-shock-protein-70 induction following pulsed laser irradiation in a transgenic reporter mouse. O'Connell-Rodwell, CE, Mackanos, MA, Simanovskii, D, Cao, Y-A, Bachmann, MH, Schwettmann, HA, Contag, CH (2008) **J Biomed Opt.** 13(3):030501.

Short-duration-focused ultrasound stimulation of Hsp-70 expression in vivo. Kruse, D. E., Mackanos, M. A., O'Connell-Rodwell, C. E., Contag, C. H., Ferrara, K. W. (2008) **Phys Med Biol**. 53(13): 3641-3660.

Accelerated Bone Repair After Plasma Laser Corticotomies. Philipp Leucht, Kentson Lam, Jae-Beom Kim, Mark A. Mackanos, Dmitrii M. Simanovskii, Michael T. Longaker, Christopher H. Contag, H Alan Schwettman, and Jill A. Helms, Ann Surg. 2007 July; 246(1): 140–150.

Cellular Tolerance to Pulsed Hyperthermia. D.M. Simanovskii, M.A. Mackanos, A.R. Irani, C.E. O'Connell-Rodwell, C.H. Contag, H.A. Schwettman, and D.V. Palanker. *PHYSICAL REVIEW E*, **74(1)**, 011915: 1539-3755 (2006)

A Genetic Reporter of Thermal Stress Defines Physiologic Zones Over a Defined Temperature Range. C.E. O'Connell-Rodwell, D.Shriver, D.M. Simanovskii, C. McClure, Y. Cao, W. Zhang, M.H. Bachmann, J.T. Beckham, E.D. Jansen, D. Palanker, H.A. Schwettman, C.H. Contag; *FASEB J.*, **18**: 264-271 (2004).

Prevention of tissue damage by water jet during cavitation. D. Palanker, A.Vankov, J. Miller, M. Friedman, and M. Strauss*; Journal of Applied Physics*, **94(4):** 2654-2661 (2003).

c) Conference Proceedings Publications:

Tissue Dissection with Ultrafast Laser using Extended and Multiple Foci. I. Toytman, A. Silbergleit, D. Simanovski, D. Palanker, Optical Interactions with Tissues and Cells XXI, SPIE vol. 7562 (2010).

Cellular tolerance to pulsed heating. D. Simanovskii, M. Sarkar, A. Irani, C. O'Connell-Rodwell, C. Contag, A. Schwettman, D. Palanker. *Optical Interactions with Tissue and Cells XVI, SPIE* vol. **5695** (2005).

Pulsed Liquid Microjet for Intravascular Injection, D. Palanker, D. Fletcher, P. Huie, J. Miller, M. Marmor, M. Blumenkranz, *Ophthalmic Technologies,* vol. **4611,** SPIE (2002).

Effect of the Probe Geometry on Dynamics of Cavitation, D. Palanker, A. Vankov, J. Miller, *Laser-Tissue Interactions XIII,* vol. **4617** SPIE (2002).

Pulsed Liquid Microjet for Microsurgical Applications, D. V. Palanker, D. A. Fletcher, *Novel Micro- and Nanotechnologies for Bioengineering Applications (**BO35**), SPIE (2001).*

4. Pulsed Electron Avalanche Knife

a) Significant Accomplishments:

Electrosurgery, one of the most common surgical tools, has changed surprisingly little since its invention almost a century ago. Continuous radiofrequency is still used for tissue cutting, with thermal damage extending to hundreds of micrometers. In contrast, lasers developed 70 years later, have been constantly perfected, and the laser-tissue interactions explored in great detail, which has allowed tissue ablation with cellular precision in many laser applications. We studied the mechanisms of interactions of electric discharges with biological tissues and associated tissue damage. We developed a pulsed plasma-mediated approach to electrosurgery, and demonstrate that with properly optimized waveforms and microelectrodes electrosurgery can rival many advanced lasers. Pulsed electric waveforms with burst durations ranging from 10 to 100 ms applied via insulated planar electrodes with 10 mm exposed edges dissects tissues with the collateral damage zone not exceeding a single cell: from 2 to 10mm. Length of the electrodes can vary from micrometers to centimeters and all types of soft tissues—from membranes to cartilage and skin could be dissected in liquid medium and in a dry field. This technology allowed for major improvements in multiple fields: plastic, intraocular, cardiac, ENT, orthopedics, and others. This device, manufactured now by a company called PEAK Surgical, is approved for clinical use world-wide.

b) Peer-Reviewed publications (in reversed chronological order):

Anterior Capsulotomy with a Pulsed Electron Avalanche Knife (PEAK). D. Palanker, H. Nomoto, P. Huie, A. Vankov, D.F. Chang. *Journal of Cataract and Refractive Surgery,* **36(1)**: 127-132 (2010)

Comparative Healing of Surgical Incisions Created by the PEAK PlasmaBlade, Conventional Electrosurgery, and a Scalpel. S.A. Loh, G.A. Carlson, E.I. Chang, E. Huang, D. Palanker, G.C. Gurtner. *Plastic and Reconstructive Surgery*, **124 (6)**: 1849-1859 (2009).

On Mechanisms of Interaction in Electrosurgery. D. Palanker, A. Vankov, P. Jayaraman. *New Journal of Physics*. **10**: 123022 (15pp) (2008).

Electrosurgery with Cellular Precision. D. Palanker, A. Vankov, P. Huie. *IEEE Transactions on Biomedical Engineering*, **55(2)**: 838-841 (2008).

Pulsed electrical stimulation for control of vasculature: Temporary vasoconstriction and permanent thrombosis. D. Palanker, A. Vankov, Y. Freyvert, P.Huie, *Bioelectromagnetics*, **29**:100-107 (2008).

Nanosecond plasma-mediated electrosurgery with elongated electrodes. A. Vankov, D. Palanker, *Journal of Applied Physics*, 101: 124701 (2007)

Pulsed Electron Avalanche Knife (PEAK-fc): New Technology for Cataract Surgery. S.G. Priglinger, D. Palanker, C.S. Alge, T.C. Kreutzer, C. Haritoglou, M. Grueterich and A. Kampik, *British Journal of Ophthalmology,* **91**: 949 — 954 (2007).

Gene Transfer to Rabbit Retina with Electron Avalanche Transfection. T.W. Chalberg, A. Vankov, F.E. Molnar, A.F. Butterwick, P. Huie, M.P. Calos, and D.V. Palanker, *Investigative Ophthalmology and Visual Science* **47**: 4083-4090 (2006).

Pulsed electron avalanche knife for capsulotomy in congenital and mature cataract. Priglinger, SG; Haritoglou, C; Palanker, D; Kook, D; Grueterich, M; Mueller, A; Alge, CS; Kampik, A. *Journal of Cataract and Refractive Surgery*; **32(7)**: 1085-1088 (2006).

Pulsed Electron Avalanche Knife (PEAK-fc) for Dissection of Retinal Tissue. S.G. Priglinger, C. Haritoglou,, D. Palanker, C. Alge, A. Gandorfer, A. Kampik, *Archives of Ophthalmology*, **123 (10)**: 1412-1418 (2005).

Pulsed Electron Avalanche Knife in Vitreoretinal Surgery. S.G. Priglinger, C. Haritoglou, A. Mueller, M. Grueterich, R. Strauss, C.S. Alge, A. Gandorfer, D. Palanker, A. Kampik, *Retina*, **25(7)**: 889-896 (2005).

Precision and Safety of the Pulsed Electron Avalanche Knife in Vitreoretinal Surgery. J. Miller, D. Palanker, A. Vankov, M. Marmor, M. Blumenkranz, *Archives of Ophthalmology,* **121**: 871-877**,** 2003.

Intra-vascular drug delivery with a pulsed liquid microjet. D. A. Fletcher, D. V. Palanker, P. Huie, J. Miller, M.F. Marmor, M. S. Blumenkranz, *Archives of Ophthalmology,* **120(9)**: 1206-1208, 2002.

Effects of the Pulsed Electron Avalanche Knife (PEAK) on Retinal Tissue. D.V. Palanker, M.F. Marmor, A. Branco, P. Huie, J.M. Miller, S.R. Sanislo, A. Vankov, M.S. Blumenkranz, *Archives of Ophthalmology,* **120:**636-640, 2002.

Pulsed Liquid Microjet For Microsurgery, D. A. Fletcher, D. V. Palanker, *Applied Physics Letters,* 78(13): 1933-35, 2001.

Pulsed Electron Avalanche Knife for Intraocular Surgery, D.V. Palanker, J.M. Miller, S.R. Sanislo, M.F. Marmor, M.S. Blumenkranz, *Investigative Ophthalmology and Visual Science,*, **42(11)**: 2673-2678, 2001.

c) Conference Proceedings Publications:

Plasma-Mediated Transfection of RPE. D. Palanker, T. Chalberg, A. Vankov, P. Huie, F.E. Molnar, A. Butterwick, M. Calos, M. Marmor, M.S. Blumenkranz. *Ophthalmic Technologies XVI,* SPIE vol. **6138** (2006).

Electro-adhesive forceps for tissue manipulation. A. Vankov, P. Huie, M.S. Blumenkranz, D. Palanker. *Ophthalmic Technologies,* vol.**5314,** SPIE (2004).

Optimization of the Pulsed Electron Avalanche Knife for Anterior Segment Surgery. D. Palanker, A.Vankov, K.Bilbao, M.Marmor, M.Blumenkranz, *Ophthalmic Technologies***,** SPIE, vol. **4951:** 56-61, (2003).

d) Agency or corporate funding spawned by project accomplishments:

Peak Surgical Inc. – Research grant through Stanford Photonics Research Center. 2006-2007.

e) Patents:

US 7,789,879 System for plasma-mediated thermo-electrical surgery
US 7,736,361 Electrosurgical system with uniformly enhanced electric field and minimal collateral damage
US 7,357,802 Electrosurgical system with uniformly enhanced electric field and minimal collateral damage
US 7,238,185 Method and apparatus for plasma-mediated thermo-electrical ablation
US 6,913,605 Microfluidic devices and methods for producing pulsed microfluidic jets in a liquid environment
US 6,780,178 Method and apparatus for plasma-mediated thermo-electrical ablation

All patents of this technology are assigned to Stanford University Office of Technology Licensing, and licensed to PEAK Surgical Inc.

5. Ophthalmology

a) Significant Accomplishments:

We have studied interactions of lasers with various ocular tissues in a broad range of pulse durations and wavelengths. We have developed and verified a quantitative model of thermal tissue damage. This insight allowed for improvement in safety of retinal photocoagulation using spatial and temporal modulation of the laser beam. We have discovered retinal plasticity following laser damage, which involves migration and rewiring of the photoreceptors from the adjacent

undamaged areas. This phenomenon could be used for restoration of photoreceptor layer following retinal damage. We have developed a Pattern Scanning Laser system (PASCAL) for ocular photocoagulation, which is now used world-wide for retinal and glaucoma therapy (was initially manufactured by OptiMedica and recently sold to Topcon). We have developed scanning approach to selective treatment of retinal pigment epithelium, which avoids damage to photoreceptors. This is achieved by very rapid scanning of a continuous laser, with a beam dwell time in microseconds. We have found a range of thermal cellular stimulation below the lethal threshold, which may allow for sub-lethal retinal laser therapy. We currently study the effects of UV and multiphoton interactions with transparent ocular tissues, with applications for repair of the corneal thermal damage.

b) Peer-Reviewed publications (in reversed chronological order):

Femtosecond Laser–Assisted Cataract Surgery with Integrated Optical Coherence Tomography. D. V. Palanker, M. S. Blumenkranz, D. Andersen, M. Wiltberger, G. Marcellino, P. Gooding, D. Angeley, G. Schuele, B. Woodley, M. Simoneau, N. J. Friedman, B. Seibel, J. Batlle, R. Feliz, J. Talamo, W. Culbertson,. *Science Translational Medicine* **2**, 58ra85 (2010).

Non-damaging Retinal Phototherapy: Dynamic Range of Heat Shock Protein Expression. C. Sramek, M. Mackanos, R. Spitler, L.S. Leung, H. Nomoto, C. Contag, D. Palanker. *Invest. Ophthalmol. Vis. Sci.* published online ahead of print 18 November 2010, 10.1167/iovs.10-5917

Selective Retinal Therapy with Microsecond Exposures Using a Continuous Line Scanning Laser. Y. M. Paulus, ATul Jain, H. Nomoto, C. Sramek, R. F. Gariano, D. Andersen, G. Schuele, L.S. Leung, T. Leng, D.l Palanker. *RETINA* :**1–9**, (2010)

Patterned Laser Trabeculoplasty . M. Turati, F. Gil-Carrasco, A. Morales, H. Quiroz-Mercado, D. Andersen, G. Marcellino, G. Schuele, D. Palanker. *Ophthalmic Surgery Lasers and Imaging*, **41**:538-545 (2010).

Short-pulse Laser Treatment: Redefining Retinal Therapy. Y. Paulus, D. Palanker, M.S. Blumenkranz. *Retinal Physician*, **7(1):** 54-59 (2010).

Dynamics of Retinal Photocoagulation and Rupture. C. Sramek, Y. Paulus, H. Nomoto, P. Huie, J. Brown, D. Palanker. *J. Biomedical Optics,* **14(3)**, 034007 (2009).

Healing of Retinal Photocoagulation Lesions. Y.M. Paulus, A. Jain, R.F. Gariano, B.V. Stanzel, M.F. Marmor, M.S. Blumenkranz, and D.V. Palanker. *Investigative Ophthalmology and Visual Science*; **49(12)**: 5540-5545 (2008).

Effect of Pulse Duration on Size and Character of the Lesion in Retinal Photocoagulation. A. Jain, M.S. Blumenkranz, Y. Paulus, M.W. Wiltberger, D.E. Andersen, P. Huie, D. Palanker. *Archives of Ophthalmology* **126 (1)**: 78-85 (2008).

Semi-Automated Pattern Scanning Laser for Retinal Photocoagulation. M.S. Blumenkranz, D. Yellachich, D.E. Andersen, M.W. Wiltberger, D. Mordaunt, G.R. Marcellino, D. Palanker, *Retina*, **26(3)**: 370-376 (2006).

The Chick Chorioallantoic Membrane (CAM) as a Model Tissue for Surgical Retinal Research and Simulation. T. Leng, J.M. Miller, K.V. Bilbao, D.V. Palanker, P.H., and M.S. Blumenkranz; *Retina,* **24 (3)**: 427-434 (2004).

c) Conference Proceedings Publications:

Selective retinal therapy with a continuous line scanning laser. Y.M. Paulus, ATul Jain, R.F. Gariano, H. Nomoto, G. Schuele, C. Sramek, R. Charalel, D. Palanker. *Ophthalmic Technologies XX, SPIE*, vol. 7550 (2010).

Improved Safety of Retinal Photocoagulation with a Shaped Beam and Modulated Pulse. C. Sramek, J. Brown, Y.M. Paulus, H. Nomoto, D. Palanker. *Ophthalmic Technologies XX, SPIE*, vol. 7550 (2010).

Computational model of retinal photocoagulation and rupture, C. Sramek, Y. Paulus, H. Nomoto, P. Huie, Daniel Palanker; *Ophthalmic Technologies XIX, SPIE*, vol. 7163 (2009).

Book Chapter:

Retinal Laser Therapy: Biophysical Basis and Applications, D. Palanker, M.S. Blumenkranz, J.J. Weiter; Chapter 22 in RETINA, 4th edition, Ed. S.J. Ryan, vol. 3, Mosby, Inc., St. Louis, MI, 2005.

d) Agency or corporate funding spawned by project accomplishments:

OptiMedica Corp. – Research grant via Stanford Photonics Research Center. 2006 – 2010.

e) Patents:

US 7,766,903 Patterned Laser Treatment of the Retina

6. Retinal Prosthesis

a) Significant Accomplishments:

Electronic retinal prostheses seek to restore sight to patients blinded by laser damage or retinal degenerative disease. Implanted electrode arrays apply patterned electrical stimulation to surviving retinal neurons, producing visual sensations. We designed and tested a photovoltaic retinal prosthesis fabricated with a pixel density of up to 256 pixels/mm^2. Photodiodes within each pixel of the subretinal array directly convert light to stimulation current, avoiding the use of bulky coil implants, decoding electronics, and wiring, and thereby reducing surgical complexity. A goggles-mounted camera captures the visual scene and transmits the data stream to a pocket processor. The resulting images are projected into the eyes by video goggles using pulsed, near infrared (~900 nm) light. In vitro tests of the photovoltaic retinal stimulation have recorded stimulated spikes from the ganglion with peak irradiance stimulation thresholds varying from 0.1 to 1 mW/mm^2. With 1ms pulses at 25Hz the average irradiance is more than 100 times below the IR retinal safety limit. Elicited retinal response disappeared upon the addition of synaptic blockers, indicating that the inner retina is stimulated rather than the ganglion cells directly, and raising hopes that the prosthesis will preserve some of the retina's natural signal processing.

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Optoelectronic retinal prosthesis: system design and performance. J.D. Loudin, D.M. Simanovskii, K. Vijayraghavan, C.K. Sramek, A.F. Butterwick, P. Huie, G.Y. McLean, and D.V. Palanker. *Journal of Neural Engineering*, **4:** S72—S84 (2007).

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Migration of Retinal Cells through a Perforated Membrane: Implications for a High-Resolution Prosthesis. D. Palanker, P. Huie, A. Vankov, R. Aramant, M. Seiler, H. Fishman, M. Marmor, M.S. Blumenkranz; *Investigative Ophthalmology and Visual Science,* **45(9)**: 3266-3270 (2004).

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Book Chapters:

Delivery of Information and Power to the Implant, Integration of the Electrode Array with the Retina, and Safety of Chronic Stimulation. J. Loudin, A. Butterwick, P. Huie, and D. Palanker. , Chapter 7 in VISUAL PROSTHETICS: Physiology, Bioengineering, Rehabilitation. G. Dagnelie (Editor), Springer 2010.

High-Resolution Electronic Retinal Prosthesis: Physical Limitations and Design. D. Palanker, A. Vankov, P. Huie, A. Butterwick, I. Chan, M.F. Marmor and M.S. Blumenkranz; Chapter 14 in ARTIFICIAL SIGHT: BASIC RESEARCH, BIOMEDICAL ENGINEERING, AND CLINICAL ADVANCES; M.S. Humayun, J.D. Weiland, G. Chader, E. Greenbaum (Eds.), Springer Series: Biological and Medical Physics, Biomedical Engineering, New York, 2007.

d) Agency or corporate funding spawned by project accomplishments:

NIH R01 grant 1R01-EY-018608 “High Resolution Photovoltaic Retinal Prosthesis”, 2009-2013.
Stanford Bio-X IIP grant “Optoelectronic Retinal Prosthesis” 2008 – 2009.

e) Patents:

US 7,447,547 Neural prosthesis based on photomechanical deflectors and tactile sensory cells
US 7,058,455 Interface for making spatially resolved electrical contact to neural cells in a biological neural network
US 7,047,080 Self-sufficient retinal prosthesis powered by intraocular photovoltaic cells

7. Molecular Imaging with Reporter Genes

a) Significant Accomplishments: The origins of in vivo bioluminescence imaging and the use of reporter genes to mark cellular and molecular events and track them in vivo began with this

funding from the DoD. This advance was one of the key events that led to the new field of "Molecular Imaging". The set of tools that originated with this funding has evolved into a versatile and broadly applicable imaging modality that comprises the fundamental bridged between cell cultures assays and the in vivo study of cellular and molecular events in living animals. As such these tools have revolutionized preclinical studies of regenerative medicine, infection/immunity, stem cell biology, oncology, and physiology. We now have "transparent" animal models of human biology and disease that can be used to accelerate and refine the study of biology leading to a greater understanding of mammalian biology, and significant improvements in drug development. This basic technology is now used by every large drug company in the world and many smaller biotechnology companies to accelerate drug discovery and development. The are over 1000 instruments for in vivo bioluminescence imaging, world-wide, that have been placed in academic and commercial laboratories that are being used to study mammalian biology. There are over 40 new drugs and formulations that have been presented to the FDA using in vivo bioluminescene imaging data to support their applications. At Stanford, we are using these technologies to study biology in a large number of fields.

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c) Conference Proceedings Publications: Too numerous to list

d) Agency or corporate funding spawned by project accomplishments:

Current funding:

1. In vivo Imaging of Hypoxia-Inducible Physiology

NIH: 1 R21 EB005442-01A2(Graves) 06/01/07 - 05/31/2010

In this project we will engineer novel reporter genes for the detection of hypoxia-induced gene transcription and translation, and use these to study signaling pathways in hypoxia-directed cancer treatments.

2. Center for Biophotonics Science and Technology

NSF – UC, Davis; 002865-SU (Matthews) 08/01/2008–07/31/2009

The mission of the center is to support multi-disciplinary research in biophotonics. In our project will advance bioassays for the early detection and staging of prostate cancer.

3. Multi-Modality Cellular Imaging of Vascular Inflammation

NIH/NHLBI; 1R01 HL 078678-03 (McConnell) 09/22/2004–05/31/2009

The aim of this project is to study the pathogenesis of atherosclerosis using a multimodality imaging approach.

4. In Vivo and Molecular Imaging Center @ Stanford

NIH/NCI; P50 CA 114747-02 (Gambhir) 08/01/2005–06/30/2010

Project 2. The aims of the center are to provide better links between pre-clinical models of cancer and the clinical management of cancer patients by novel research in multimodality molecular imaging.

5. Bone Marrow Grafting for Leukemia and Lymphoma

NIH/NCI; 2 P01 CA49605-19 (Negrin) 04/01/2002–02/29/2009

Core C: Imaging/Molecular Pathology. This program project grant supports experimental and clinical studies concerning allogeneic and autologous bone marrow or peripheral blood progenitor cell transplantation for leukemia, Hodgkin's disease, non-Hodgkin's lymphoma and multiple myeloma.

6. Nanoparticle Formation and Biodistribution for Optimizing Therapy

NIH; 1 R21 CA125467 (Zare) 01/01/2007-12/31/2009

In this project we will develop supercritical antisolvent processes to form nanoparticles and use these particles will be used to encapsulate imaging agents or drugs for targeted delivery to tumors.

7. Cloning and Engineering the IC2 Biomarker Targeting Functional Beta Cells

MilliGen (Contag) 9/01/2008-08/31/2010

The aim of this proposal is the molecular engineering of genes encoding the rat monoclonal IC2 biomarker to develop a bioluminescent biomarker targeting insulin secreting functional human beta cells in vitro and in vivo.

8. Delivery of Biologically Active Nucleic Acid to Epidermal Cells

NIH; Transderm(Contag) 10/01/2009-09/30/2011

9. Engineering embryonic stem cells for operational tolerance

CIRM; RM1-01706 10/01/2010-09/31/201

The objective of this project is to identify a set of genes that can modify the immune response towards embryonic stem cells such that a state of sustained tolerance is achieved.

<u>Past funding:</u>

Small Animal Imaging Core Resource. Stanford University Program in Biomedical Engineeringand Child Health Initiative-Lucille Packard Foundation. (Principal Investigators: C. H. Contag)

Mobilized dendritic cells for lung cancer, NIH; PO1HL57443-06 (Principal Investigator: E. Engleman)

Regulation of heme oxygenase in neonatal animals, NIH/NICHD; RO1 HL58013 (Principal Investigators: D.K. Stevenson and C.H. Contag)

Monitoring tumor progression in living animals, NIH/NCI; RO1 CA80006-01 (Principal Investigators: R.S. Negrin and C.H. Contag)

Therapeutic Use of Heme Analogs: Absorption in Intestine, NHLB/NIH; RO1 HL 68703 (Principal Investigators: D.K. Stevenson and C.H. Contag)

Spatiotemporal analysis of neoplastic disease. NCI/NIH; R24 CA 92862 (Principal Investigators: C. H. Contag, M. Bednarski, M. Moseley)

Visible Models of Neoplastic Disease, NIH/NCI; R33 CA88303 (Principal Investigators: C.H. Contag and R.S. Negrin)

Visualizing tumor progression and therapy in living animals, Leukemia Society ; 6090-99 (Principal Investigators: R.S. Negrin and C.H. Contag)

Oral immunization against HIV using attenuated salmonella strains as gene delivery vehicles; (Stanford, CHI-Packard Foundation; Principal Investigator: C. H. Contag)

Imaging Spontaneous Breast Cancer in Mouse Models, NIH/NCI ; R21 CA87386-01A1 (Principal Investigators: C.H. Contag and R.S. Negrin)

In vivo multimodality Imaging of Neoplastic Disease, NCI/NIH
P20 CA86312-01 (Principal Investigators: C. H. Contag, M. Moseley, M Bednarski)
R24 HD37543 (Principal Investigators: C.H. Contag and D.K. Stevenson)

Visualizing insulitis: IDDM pathogenesis and therapy, NIH/NIDDK ; RO1 DK58664 (Principal Investigators C.H. Contag and C.G. Fathman)

Novel Cancer Targeting Mechanism for Imaging with PE; DOD
WH-05-1-0059 (Principal Investigator: B. Franc, UCSF)

Gene therapy of IDDM and its complications, JDFI; 4-2001-9, JDFI Center Grant (Principal Investigator: C. G. Fathman)

Spatiotemporal analysis of neonatal host response, NIH/NICHD-NIAID

e) Patents:

US Pat. 7255851. Filing date: Jun 2, 2005; Issue date: Aug 14, 2007; Non-invasive localization of a light-emitting conjugate in a mammal. C.H. Contag, P.R. Contag and D.A. Benaron. Detecting and tracking pathogens in living hosts.

C.H. Contag, P.R. Contag and D.A. Benaron. (1996) Ligand targeted biodetectors. Corporate owner: Xenogen Corporation. Issued.

C.H. Contag, and B.F. Eames (2000) Red-shifted Luciferase. Corporate owner: Stanford University. Issued.

8. In vivo Imaging: Advanced Microscopy

a) Significant Accomplishments: Based on a dual axis design we have built a multispectral, miniaturized confocal microscope that can be implanted at sites of injury and used to assess therapeutic outcome. The self-aligning system is simple and robust and can be used to refine these studies and accelerate the analyses. The improved multispectral optical design of the MEMS-based dual-axis fiber optic confocal scanning microscope has been miniaturized for in vivo imaging of tissues, and which is also capable of being further scaled to smaller dimensions. Based on the principles of conic sections, we use a parabolic mirror to focus two parallel collimated beams to a common point inside the tissue. The simplicity of the design requires only a small number of optical components that are assembled within the small and rugged cylindrical housing and allows self-alignment of the microscope. In addition, a second parabolic mirror is used to provide the two parallel collimated beams, which are then focused to a common point by the "focusing mirror". This combination of two parabolic mirrors, which are facing each other, provides a simple dual-axis confocal (DAC) design that is initially self-aligning during the assembly of the microscope, and which also remains properly aligned over a wide range of wavelengths. The parabolic mirrors are incorporated into glass catadioptric lenses that are mounted and sealed at each end of the stainless steel microscope. In addition to the self-aligning properties of this system, the achromatic optical design of the catadioptric lenses can thus provide multispectral reflectance or fluorescence images that are simultaneously produced by a scanning illumination beam comprising different wavelengths. It is important in this case for the images produced at different wavelengths to be all co-registered to each other at the same working distance (depth) in the tissue and over the same scanned FOV. This dual-parabolic mirror design provides such improved achromatic performance. The scanning and de-scanning respectively, of the illumination and collection beams in this design is similar to previous DAC microscopes. As in previous designs, a MEMS scanner is used for high speed scanning in the X-Y plane below the tissue surface at a set working distance. An additional feature, also used in previous DAC systems, is a mechanism for controlling a variable working distance, thus producing a scan in the Z direction and allowing capture of 3-D volumetric images of tissue. The design parameters that affect the resolution, FOV, and working distance have been analyzed for previous DAC systems using Zemax™ and FRED™ optical modeling software and have been verified by experimental results. Other design features that have been used in previous DAC systems include the use of a solid immersion lens (SIL) having a low refractive index close to that of tissue, which provides an index-matched optical interface into the tissue, thereby enhancing the resolution, flattening the imaging field, and minimizing the aberrations that usually occur at the glass-tissue interface.

b) Peer-Reviewed publications (in chronological order):

Wang, TD, Contag, CH, Mandella, MJ, Chan, NY, Kino, GS (2003) Dual axes confocal microscopy with post objective scanning and low-coherence heterodyne detection. Optics Letters 28(20): 1915-1917

Wang, TD, Mandella, MJ, Contag, CH and Kino, GS (2003) Dual-Axes Confocal Microscope for High Resolution *In Vivo* Imaging. Optics Letters. 28(6): 414-6.

Wang, TD, Mandella, MJ, Contag, CH, Kino, GS (2004) Dual Axes Confocal Fluorescence Microscope for *In Vivo* Molecular and Cellular Imaging. J. Biomed Optics. 9:735-742.

Wang, TD, Friedland, S, Sahbaie, P, Soetikno, R, Hsiung, P-L, Liu, JTC, Crawford, JM, Contag, CH. (2007) Functional imaging of colonic mucosa with a fibered confocal microscope for real time in vivo pathology. Clin Gastro Hepatol. 5(11) 1300-5

Wang, TD, Triadafilopoulos, G, Crawford, JM, Dixon, LR, Bhandari, T, Sahbaie, P, Friedland, S, Soetikno, R, Contag, CH. (2007) Detection of Endogenous Biomolecules in Barrett's Esophagus by Fourier Transform Infrared Spectroscopy. Proc. Natl. Sci, USA. 104(40): 15864-9.

Liu JT, Mandella MJ, Ra H, Wong LK, Solgaard O, Kino GS, Piyawattanametha W, Contag CH, Wang TD (2007) Miniature near-infrared dual-axes confocal microscope utilizing a two-dimensional microelectromechanical systems scanner. Opt Lett 32(3):256-8

Hsiung, P-L, Hardy, JW, Friedland, S, Soetikno, R, Du, CB, Wu, APW, Sahbaie, P, Crawford, JM, Lowe, AW, Contag, CH, Wang, TD. (2008) Detection of colonic dysplasia *in vivo* using a targeted fluorescent septapeptide and confocal microendoscopy. Nat. Med. 14(4): 454-8.

Ra, H, Piyawattanametha, W, Mandella, MJ, Hsiung, P-L, Hardy, J, Wang, TD, Contag, CH, Kino, GS, and Solgaard, O. (2008) Three-dimensional *in vivo* imaging by a handheld dual-axes confocal microscope. Optics Express. 16(10): 7224-7232.

Liu, JT, Mandella, MJ, Crawford, JM, Contag, CH, Wang, TD, Kino, GS. (2008) Efficient rejection of scattered light enables deep optical sectioning in turbid media with low-numerical-aperture optics in a dual-axis confocal architecture. J Biomed Opt. 13(3):034020.

Mackanos, MA, Hargrove, J, Wolters, R, Du, CB, Friedland, S, Soetikno, RM, Contag, CH, Arroyo, MR, Crawford, JM, Wang, TD (2009) Use of an endoscope-compatible probe to detect colonic dysplasia with Fourier transform infrared spectroscopy. J Biomed Optics 14, 044006. PMID: 19725718

Liu JT, Helms MW, Mandella MJ, Crawford JM, Kino GS, Contag CH (2009) Quantifying cell-surface biomarker expression in thick tissues with ratiometric three-dimensional microscopy. Biophys J. 96:2405-2414.

Liu JT, Mandella MJ, Loewke NO, Haeberle H, Ra H, Piyawattanametha W, Solgaard O, Kino GS, Contag CH. (2010) Micromirror-scanned dual-axis confocal microscope utilizing a gradient-index relay lens for image guidance during brain surgery. J Biomed Opt. 15 (2): 026029

Loewke, KE, Camarillo, DB, Piyawattanametha, W, Mandella, MJ, Contag, CH, Thrun, S, Salisbury, JK (2010) *In Vivo* Micro-image Mosaicing. IEEE Trans Biomed Engineering. 58(1): 159-171.

Mackanos MA, Contag CH. (2010) Fiber-optic probes enable cancer detection with FTIR spectroscopy. Trends Biotechnol. 28(6):317-23.

Mackanos MA Contag CH. (2009) FTIR microspectroscopy for improved prostate cancer diagnosis. Trends Biotechnol. 27(12): 661-663. PMID: 19853940.
Mackanos, MA, Jansen, ED, Contag, CH. (2011) Molecular Imaging Using Fluorescence and Bioluminescence to Reveal Tissue Response to Laser-Mediated Thermal Injury. In: Optical-Thermal Response of Laser-Irradiated Tissue (Ed. Welch and Gemert) pp 799- 823.
Liu, JTC, Hardy, JW, Contag, CH. (2011) High-resolution c**onfocal endomicroscopy for gastrointestinal cancer detection. In:** Advances in Optical Imaging for Clinical Medicine. (eds. Iftimia, Brugge and Hammer) Wiley Press. pp .
Hardy, JW, Liu, JTC, Lowe, AW, Contag, CH. (2011) Molecular probes for optical contrast enhancement of gastrointestinal cancers. **In:** Advances in Optical Imaging for Clinical Medicine. (eds. Iftimia, Brugge and Hammer) Wiley Press. pp 505-527.
Contag, CH (2007) In vivo Pathology: Seeing with molecular sensitivity and cellular resolution in the living body. Annu. Rev. Pathol. Mech. Dis. 2:277–305

c) Conference Proceedings Publications: To numerous to list.

d) Agency or corporate funding spawned by project accomplishments:
Multimodality Imaging of GI Cancers for Diagnosis and Directed Therapy, NIH/NCI
U54 CA136465-01 (Principal Investigator: C.H. Contag)
Use of dual axes confocal microscopy to visualize tumor margins in medulloblastoma
Children's Brain Cancer Center (Principal Investigator: C.H. Contag)
Detection of neoplasia in the esophagus, NIH/NCI
U54 CA105296-01 (Principal Investigator: C.H. Contag)

e) Patents:

9. Liposome-Mediated Delivery of Wnt

Significant Accomplishments: Modern armor often spares the lives of warfighters, but it does not protect limbs and craniofacial tissues from severe blast injuries. As a result, amputations, a last resort following unsuccessful attempts of initiating a healing response, account for almost 3% of all wounded in the Iraq war. We have advanced an "encapsulate and activate" approach that allows for the delivery of biomolecules when and where they are needed to stimulate tissue healing. The thermal activation of cellular processes is being used to establish functional changes in the damaged tissues to promote healing and direct the regenerative process in soft tissues such as muscle and skin. Laser-mediated control of drug delivery will be developed for local release of regulatory molecules that mediate regeneration of damaged tissues. We encapsulate the biomolecules in lipid vesicles such that biological activity is maintained even in the protease-rich environment of a wound site. We have investigated strategies to selectively activate the lipid-encapsulated biomolecules using short pulses of near-infrared laser light for directed delivery of biomolecules and controlled tissue healing. With the goal of eliminating scaring and fibrosis at the skin wound site, which is a mandatory first step for skin regeneration to occur. We have used a key growth factor, Wnt3a, which stimulates the proliferation and differentiation of tissue-specific epidermal stem cells, to accelerated skin wound healing. We generated Wnt liposomes with biological activity and test for their ability to accelerate skin wound healing via activation of epidermal stem cells. In natural tissue repair, Wnt is present for only limited periods of time without carcinogenic effect. Our application of Wnt mimics the normal transient burst of Wnt

signaling seen after tissue injury, and therefore is highly unlikely to pose carcinogenic risk. We have tested this prediction by examining the local and systemic effects resulting from the delivery of liposomal Wnt using reporter mice to monitor activation of the Wnt pathway coupled with live imaging to track the distribution and *in vivo* longevity of liposomes.

b) Peer-Reviewed publications (in reversed chronological order):

Laser-induced disruption of systemically administered liposomes for targeted drug delivery. Mackanos, MA, Larabi, M, Shinde, R, Simanovskii, DM, Guccione, S, Contag, CH (2009) **J Biomed Optics** 14(4): 044009.

Controlling the In Vivo Activity of Wnt Liposomes. Chapter 17 ; L. Zhao, S.M. Rooker, N. Morrell, P. Leucht, D. Simanovskii and J.A. Helms; Methods in Enzymology; Volume 465, 2009, Pages 331-347

c) Conference Proceedings Publications:

d) Agency or corporate funding spawned by project accomplishments:

e) Patents:

10. Restoration of Hearing

a) Significant Accomplishments: Hearing loss is a key health problem afflicting military personnel and the general public. Our work has been aimed at improved therapeutic approaches and novel prosthetic technologies for treating hearing loss due to inner ear damage or pathology. For this purpose we have advanced microscope designs for studying living cells and tissue in the context of intact organs, and here focus on the inner ear. The high-speed two-photon fluorescence microscope was designed such that the entire imaging field can be illuminated with a single high-energy pulse from a regenerative laser amplifier, split between 10^4-10^5 foci by a micro-lens array in each image frame. This accelerates data acquisition while retaining resolution. This is used to develop general-purpose, clinical instrumentation for fluorescence microendoscopy and a detachable probe specially designed for use in the human cochlea. This microscope will enable minimally invasive, cellular level imaging within the inner ear. For many *in vivo* imaging applications concerning rapid biological dynamics, conventional laser-scanning two-photon fluorescence microscopy is currently limited by slow scanning speeds and frame rates. The highest frame rates that have been demonstrated to date using raster scan patterns are about 30 Hz, which is insufficient to capture biological events on the millisecond timescale. Yet, this timescale is of key importance for *in vivo* studies of electrically excitable cells, such as in the brain and heart. Although multi-focal approaches to faster two-photon imaging have been explored, these have not been widely successful for cardiac or brain imaging in live subjects due to reduced signal strengths and tissue penetration depths resulting from the division of excitation power. To overcome these challenges, we have developed a novel format for two-photon imaging that *entirely eliminates laser-scanning* and that will allow for high-speed image acquisition with frame rates up to 1000 Hz, while preserving the established benefits of two-photon imaging regarding long wavelength excitation, intrinsic optical sectioning, and reduced photodamage and photobleaching. To achieve such fast frame rates we use large microlens arrays of up to 10^4-10^5 elements, thereby providing high-density light coverage of the image field using multiple laser foci. Despite this manifold division of excitation light, we can nonetheless obtain fluorescence signal strengths comparable to those of conventional two-photon imaging by using a Ti:sapphire regenerative laser amplifier with 1 mJ

pulse energies and 5 kHz repetition rates. In this scheme the imaging frame rate is synchronized to the repetition rate of the laser amplifier, another novel feature of our design. For extending the optical penetration depth into tissue, the microscope will retain an option to scan a reduced number of foci (*e.g.* ~10^3) to attain frame rates of <1 kHz. A main challenge to attaining the high-speed imaging needed to study such fast phenomena concerns the signal-to-noise ratio, which invariably suffers in a fast serial scanning approach, since each pixel is illuminated for only microseconds. Thus, any technology aimed at improving the current limitations must address two issues: 1) how can the image be sampled quickly? and 2) how can the signal-to-noise ratio be preserved despite fast sampling?

Our microscope design involves a much larger microlens array (up to 10^4-10^5 elements) than previously employed for two-photon microscopy. This will allow us the option of eliminating laser-scanning entirely, if the user desires, since there are sufficient numbers of laser foci to fully illuminate an image of over 256 × 256 pixels, which is larger than the image sizes regularly used today for fast neuronal imaging. Although our research on this novel imaging format remains at a design stage, our work to date has involved making the above ideas quantitative through calculations comparing fluorescence excitation in our approach versus conventional two-photon microscopy. Moreover, we have also created optical designs of our microscope in CodeV, a common software package for optical ray tracing.

b) Peer-Reviewed publications (in reversed chronological order):

Wilt, B.A., Burns, L.D., Wei Ho, E.T., Ghosh, K.K., Mukamel, E.A., and Schnitzer, M.J. (2009). Advances in light microscopy for neuroscience. Annu Rev Neurosci *32*, 435-506.

Flusberg, B.A., Cocker, E.D., Piyawattanametha, W., Jung, J.C., Cheung, E.L., and Schnitzer, M.J. (2005). Fiber-optic fluorescence imaging. Nat Methods *2*, 941-950.

Piyawattanametha, W., Cocker, E.D., Burns, L.D., Barretto, R.P., Jung, J.C., Ra, H., Solgaard, O., and Schnitzer, M.J. (2009). In vivo brain imaging using a portable 2.9 g two-photon microscope based on a microelectromechanical systems scanning mirror. Opt Lett *34*, 2309-2311.

Piyawattanametha, W., Barretto, R.P., Ko, T.H., Flusberg, B.A., Cocker, E.D., Ra, H., Lee, D., Solgaard, O., and Schnitzer, M.J. (2006). Fast-scanning two-photon fluorescence imaging based on a microelectromechanical systems two- dimensional scanning mirror. Opt Lett *31*, 2018-2020.

c) Conference Proceedings Publications:

d) Agency or corporate funding spawned by project accomplishments:

e) Patents:

11. Biomatricies for wound healing

a) Significant Accomplishments: The development of novel matrices that not only provide rapid and biomimetic wound coverage, but also provide controlled local delivery of various factors that foster healing as well as antimicrobials, have been developed to advance wound healing though facilitating delivery in a time- and dose-dependent manner and on demand. In mice, we are able to study, in a controlled manner, the effects of delivering various growth factors on wound healing using advanced microscopic techniques—some of which will have utility in evaluating battlefield wounds in that they are small portable and powerful imaging devices. These approaches have not been realized previously, but the convergence of expertise in several areas and a track record of

innovation has enabled the foundation of this approach. The ability to manipulate the wound environment in a way that mimics, and goes beyond, the natural processes *in vivo* will be essential for tissue regeneration. Furthermore, our studies have contributed to the understanding of the effects of antimicrobials, HBOT, stem cell derived factor (SDF-1; a.k.a. CXCL12) in wound healing and especially to understand the consequences of the delivery of epidermal growth factor (EGF) and SDF-1, and other factors, when they are applied individually and in sequence. Because of the need for improved healing in abnormal situations, especially those that are a result of battlefield injuries, this line of investigation is significant for soldier health because it will offer a novel strategy for improving impaired healing. This is an area of critical medical need in battlefield medicine.

b) Peer-Reviewed publications (in chronological order):

Zurab, S, Scholl FA, Oliver SF, Adams A, Contag, CH, Wender, PA, Khavari, PA (2003) Gene transfer via reversible plasmid condensation with cysteine-flanked, internally spaced arginine-rich peptides. Hum Gene Ther. 1;14(13):1225-33.

Hamblin, MR, Zahra T, Contag, CH, McManus, AT, Hasan, T (2003) Optical monitoring and treatment of potentially lethal wound infections in vivo. J Infect Dis. 1;187(11):1717-25.

Cowan, CM, Shi, YY, Aalami, OO, Chou, YF, Mari, C, Romy, T, Quarto, N, Contag, CH, Wu, B, Longaker, MT (2004) Adipose-derived adult stromal cells heal critical-size mouse calvarial defects. Nat Biotechnol. 22(5): 560-567.

Cowan, CM, Aalami, OO, Shi, YY, Chou, YF, Mari, C, Quarto, TR, Nacamuli RP, Contag CH, Wu B, Longaker MT (2005) Bone morphogenetic protein 2 and retinoic acid accelerate in vivo bone formation, osteoclast recruitment, and bone turnover. Tissue Eng. 11(3-4):645-58.

Wender, PA, Goun, EA, Jones, LR, Pillow, TH, Rothbard, JB, Shinde, R, Contag, CH (2007) Real-time Analysis of Uptake and Bioactivatable Cleavage of Luciferin-Transporter Conjugates in Transgenic Reporter Mice. Proc Nat Acad Sci, USA. 104(25):10340-5.

Wang, Q, Ilves, H, Chu, P, Contag, CH, Leake, D, Johnston, BH, Kaspar, RL (2007) Delivery and inhibition of reporter genes by small interfering RNAs in a mouse skin model. J. Invest Dermatol. 127(11):2577-84.

Smith, FJ, Hickerson, RP, Sayers, JM, Reeves, RE, Contag, CH, Leake, D, Kaspar, RL, McLean, WH (2008). Development of therapeutic siRNAs for pachyonychia congenital. J Invest Dermatol 128(1): 50-8.

Gonzalez-Gonzalez, E., Ra, H., Hickerson, R.P., Wang, Q, Piyawattanametha, W, Mandella, MJ, Kino, GS, Leake, D, Avilion, AA, Solgaard, O, Doyle, TC Contag, CH. and Kaspar, RL (2009). siRNA silencing of keratinocyte-specific GFP expression in a transgenic mouse skin model. **Gene Ther.** 16:963-972

Lee, SW, Padmanabhan P, Ray P, Gambhir SS, Doyle T, Contag CH, Goodman SB, Biswal, S (2009) Stem cell-mediated accelerated bone healing observed with in vivo molecular and small animal imaging technologies in a model of skeletal injury. J Orthop Res. 27:295-302.

Jacobson GB, Shinde R, McCullough RL, Cheng NJ, Creasman A, Beyene A, Hickerson RP, Quan C, Turner C, Kaspar RL, Contag CH, Zare RN. (2010) Nanoparticle formation of organic compounds with retained biological activity. J Pharm Sci. 99 (6): 2750-5.

Franc, BL., Mandl, S, Siprashvili, Z, Khavari, P, Wender, P, Contag, CH (2003). Breaching Biological Barriers: Protein Translocation Domains as Tools for Molecular Imaging and Therapy Molec Imaging 2(4): 313-323.

c) Conference Proceedings Publications:

d) Agency or corporate funding spawned by project accomplishments:

Delivery of Biologically Active Nucleic Acid to Epidermal Cells; NIH; Subcontract through Transderm Inc. (Contag). Funding period: 10/01/2009-09/30/2011

e) Patents:

12. Two-component Hydrogels for Wound Therapy

a) Significant Accomplishments: We have created double and multi-component hydrogels. The first hydrogel component investigated consisted of recombinant protein polymers that have tailorable function and range in size from 125 to 1,000 amino acids. The protein polymers have evenly spaced reactive groups for both chemical and enzymatic crosslinking. Specifically, the protein polymers are enzymatically crosslinked into a scaffold by tissue transglutaminase (tTG). tTG creates a covalent bond between lysine and glutamine residues (each in a particular sequence context) in a mild and biocompatible reaction. The Barron laboratory has created a two-component protein polymer system, with one cationic ("K") protein that contains evenly spaced lysine substrates and a second protein that contains evenly spaced glutamine substrates for tTG ("Q"). Upon the addition of tTG enzyme, rapid crosslinking occurs to form a self-supporting hydrogel within 2 minutes. Enzymatically crosslinked protein polymer hydrogels have been shown to have low-to-zero toxicity to cells and to be degradable by cellular proteases. Using the precise control and flexibility provided by genetic engineering, protein polymers are now being created that contain matrix metalloprotease-cleavable sequence to control degradation rate, and heparin-binding domains that will sequester and release growth factors. Both of these domains are useful for tuning hydrogel properties during the wound healing process. In addition, variable scanning electron microscopy has confirmed a highly porous network, and pore size can be controlled by hydrogel composition, which is important for cellular infiltration. Protein polymers have been synthesized with heparin binding domains (HBD) that have both natural heparin binding affinity and elevated affinity. These domains will sequester heparin and subsequently growth factors for controlled delivery based on release rate. Growth factor delivery will also be dependent on enzymatic hydrogel degradation. To this end, we have created protein polymers that can both act as a substrate for tTG and also contain an MMP site for degradation. Degradation rate will be fine-tuned based on the number of MMP sites within the hydrogel. The second component of our hydrogel system are core-shell star PEGs. Star polymers are synthesized by ring opening polymerization to create a material with uniform, structurally controllable, globular, quad-functional (core, periphery, polymer, interstitial regions) nanoparticle structures. Biodegradable polycaprolactone (PCL) forms the star's core, surrounded by a hydrophilic shell formed by PEG arms. While multi-armed PEG is available commercially, the synthetic strategy we use allows the inclusion of a biodegradable PCL core and does not require heavy metal catalysis during synthesis. We can exert control over the number of arms and arm length, reactive end-groups, and molecular weight. We can make star polymers ranging in size from 20-100 nm in diameter, with anywhere from 4 to 30 arms displaying terminal chemical functionality. The functional domains allow for covalently attaching reactive groups for chemical or enzymatic cross-linking into a hydrogel. By controlling the composition and number of PEG arms, the partitioning between the core-forming and arm-forming polymers can be varied to control hydrogel properties, ultimately affecting wound healing and cell behavior.

EGF with enhanced wound healing. The practical use of EGF (like other small peptides) has been limited by their instability in physiological fluids, and a short circulation half-life of about 9-10 minutes. By using yeast cell surface display, the Cochran group has generated EGF mutant

libraries and have screened them by flow cytometry using fluorescently labeled, soluble EGFR extracellular domain to isolate EGF mutants with a 4- to 30-fold increase in receptor binding affinity. Furthermore, mutant EGF was shown to be specific to EGFR and to bind more tightly to the receptors as determined by fibroblast cell studies. In addition, the modified EGF proteins, delivered to a wound, resulted in an increased rate of healing.

Cell adhesion mediated by cystine knot peptides. The Cochran laboratory created and screened a yeast-displayed library of cystine knot peptides containing the RGD integrin recognition motif and randomized flanking residues. Engineered peptides bound to cells expressing αvβ3 integrin receptors selectively. Furthermore, the binding to αvβ3 integrin had affinities ranging from 15 nM to 780 pM.[5]

EGF delivery**.** Polymeric hydrogels can be created by a layered approach, where an initial layer will be "painted" onto wound and will contain a higher level of growth factor bound to the material. Following this, additional layers can be applied in the same manner to create a spatial distribution of growth factor. Time-dependent release of growth factor can be accomplished using biodegradable polylactic-glycolic acid (PLGA) nanoparticles to encapsulate EGF. These nanoparticles can be tailored for tunable release kinetics based on degradation rate.

Integrated delivery systems for anti-infectives and growth factors. The Barron lab has developed a new family of robust, broad-spectrum antimicrobials based on peptoid oligomers ("ampetoids"), and a number of these compounds are extremely promising. Ampetoid **1**, a 12mer containing 2/3 α-chiral (S)-*N*-(1-phenylethyl)glycine (*N*spe) and 1/3 achiral *N*-(4-aminobutyl)glycine (*N*Lys) (Fig. 12), has been found to be quite potent in its antimicrobial activity. Its structure and that of maganin-2 are shown in figure 12.

The activities of these ampetoids were compared to clinically approved AMP Pexiganan™ by measuring their minimum inhibitory concentration (MIC) against both gram-positive (*B.subtilis* ATCC 6633) and gram-negative (*E.coli* ATCC 35218) bacteria. The cytotoxicity of the ampetoids against human erythrocytes was determined by measuring the hemolytic dose (HD), the concentrations that cause heme leakage from 10% and 50% of cells. The ampetoids were essentially as active as Pexiganan, and some display high selectivity towards killing bacteria compared to human erythrocytes. Recent studies resulting from a collaboration between the Contag and Barron labs have shown that ampetoids are highly effective in preventing biofilm formation, as well as in killing biofilms that are already present.

b) Peer-Reviewed publications (in reversed chronological order):

c) Conference Proceedings Publications:

d) Agency or corporate funding spawned by project accomplishments:

e) Patents:

13. Nanoparticle delivery of antimicrobials and tissue factors to wounds.

a) Significant Accomplishments: We have developed nanoparticles of biodegradable polymers such as poly(lactic acid) (PLA) and poly(lactic-co-glycolic acid) (PLGA) with well-controlled size and surface properties. The nanoparticles will encapsulate antimicrobial drugs, siRNA and other tissue factors that assist in wound healing. Both processes are being utilized; the solution enhanced dispersion in supercritical fluids (SEDS) process and the nanoparticle precipitation process. Using predictive animal models of human biology and disease we have demonstrated controlled and sustained release with these particles.

b) Peer-Reviewed publications (in chronological order):

Jones, LR Goun, EA Shinde, R, Rothbard, JB, Contag, CH, Wender, PA. (2006) Releasable luciferin-transporter conjugates: tools for the real time analysis of cellular uptake and release. JACS. 128: 20: 6526-7

Goun, E., Shinde, R, Dehnert, K, Adams-Bond, A, Wender, PA, Contag, CH, Franc, BL (2006) Intracellular cargo delivery by an octaarginine transporter adapted to target prostate cancer cells through cell surface protease activation. Bioconjugat Chem. 17(3) 787-796

Shinde, R, Perkins, J, Contag, CH (2006) Luciferin derivatives for enhanced in vitro and in vivo bioluminescence assays. Biochem. 45(37): 11103-11112

Wender, PA, Goun, EA, Jones, LR, Pillow, TH, Rothbard, JB, Shinde, R, Contag, CH (2007) Real-time Analysis of Uptake and Bioactivatable Cleavage of Luciferin-Transporter Conjugates in Transgenic Reporter Mice. Proc Nat Acad Sci, USA. 104(25):10340-5.

Jacobson, GB, Shinde, R, Contag, CH, Zare, RN (2008) Sustained release of drugs dispersed in polymer nanoparticles. Angew Chem Int Ed Engl. 47:7880-2

Mackanos, MA, Larabi, M, Shinde, R, Simanovskii, DM, Guccione, S, Contag, CH (2009) Laser-induced disruption of systemically administered liposomes for targeted drug delivery. J Biomed Optics 14(4): 044009.

Gonzalez-Gonzalez, E., Ra, H., Hickerson, R.P., Wang, Q, Piyawattanametha, W, Mandella, MJ, Kino, GS, Leake, D, Avilion, AA, Solgaard, O, Doyle, TC Contag, CH. and Kaspar, RL (2009). siRNA silencing of keratinocyte-specific GFP expression in a transgenic mouse skin model. Gene Ther. 16:963-972

Jacobson, GB, Shinde, R, McCullough, RL, Cheng, NJ, Creasman, A, Beyene, A, Hickerson, RP, Quan, C., Turner, C, Kaspar, RL, Contag, CH, Zare, RN (2010) Nanoparticle formation of organic compounds with retained biological activity. J Pharm Sci. 99:2750-2755. PMID 20039390.

Jacobson GB, Gonzalez-Gonzalez E, Spitler R, Shinde R, Leake D, Kaspar RL, Contag CH, Zare RN. (2010) Biodegradable nanoparticles with sustained release of functional siRNA in skin. Pharm Sci. 99(10): 4261-6

Ra H, Gonzalez-Gonzalez E, Smith BR, Gambhir SS, Kino GS, Solgaard O, Kaspar RL, Contag CH. (2010) Assessing delivery and quantifying efficacy of small interfering ribonucleic acid therapeutics in the skin using a dual-axis confocal microscope. J Biomed Opt. 15 (3): 036027

c) Conference Proceedings Publications:

d) Agency or corporate funding spawned by project accomplishments:

e) Patents:

US Pat. 12469578 - Filed May 20, 2009; Encapsulated nanoparticles for drug delivery. Inventors: Christopher H. Contag, Stanford, CA (US); Rajesh R. Shinde.